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Sensitive detection of microRNA by chronocoulometry and rolling circle amplification on a gold electrode†

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We developed a quantitative detection scheme for nucleic acids, combining solid-phase rolling circle amplification and chronocoulometry (RCA–CC). A gold electrode was directly formed on a polystyrene substrate as a cost-effective and flexible biosensor for sensitive detection of microRNA (mir-143) in blood samples.

MicroRNAs, a group of small non-coding RNAs 18–25 nt in length, are widely distributed in diverse organisms and post-transcriptionally regulate protein production through mRNA cleavage or translation inhibition.[1] Over the past decade, much research has indicated that microRNAs participate in most of the cellular processes and that changes in their expression are associated with many human diseases.[2] In order to study the regulation and function of this group of small non-coding RNAs, many analytical approaches have been reported for their sensitive and specific detection.[3–6] Although northern blotting[7] and RT-PCR[8] are still regarded as golden standard techniques, many efforts have been made to develop simpler and more cost-effective methods for microRNA quantification. Electrochemical measurement is proven to be a promising method suitable for point-of-care diagnostics because of its miniaturized instrumentation, simplicity and safety.[9] However, traditional electrochemical detection could not meet the sensitivity requirement for microRNA quantification. Imperative innovations were made to improve the sensitivity of electrochemical methods including introduction of nanoparticles,[10] structure[11,12] and signal or template amplification before detection.[13–15] Sensitivity of fM to aM of target microRNA could be achieved by these innovations.

Differential pulse voltammetry (DPV) is found to be the most widely used electrochemical detection method for microRNA quantification because of its high sensitivity.[10,11,16,17] Moreover square wave voltammetry (SWV),[18] traditional cyclic voltammetry (CV)[12,19] and amperometric[14] detection were also employed as sensitive detection techniques. Chronocoulometry is a convenient electrochemical method for determination of adsorbed reactants on the electrode surface.[20] It was successfully employed to detect single stranded or duplex DNA immobilized on a gold electrode by taking advantage of the electrostatic interaction of specific redox cations with the nucleotide phosphate backbone.[21] In this report, we present a novel method for microRNA quantification by combining chronocoulometry (CC) with rolling circle amplification (RCA). The principle of this RCA–CC assay is shown in Fig. 1. An integrated three-electrode biosensor was fabricated on a polystyrene substrate by a UV directed chemical plating technique.[22] The outline of the fabrication procedure is illustrated in Fig. 1a and described in more detail in the ESI.† The working electrode (WE) was first modified with thiolated DNA probe 1 and covered with 1 mM of 6-mercapto-1-hexanol (MCH) and 1 mg mL^{-1} of BSA, respectively, to seal the excess active groups. Then target microRNA (mir-143) as well as probe 2 and a cyclized padlock probe prepared beforehand were added on the electrode surface and hybridized with probe 1. In the presence of phi29 DNA polymerase, RCA amplification occurred and replicons were measured by chronocoulometry in a low ionic strength electrolyte containing Ruhex as a redox marker.

Chronocoulometry is a promising electrochemical method for measurement of nucleic acid (*i.e.* DNA) molecules adsorbed on the surface of the electrode. Unlike other electrochemical methods, an attractive aspect of chronocoulometry is that the double layer charge and charge due to reaction of species adsorbed on the electrode surface can be differentiated from the charge due to reaction of redox molecules that diffuse to the electrode surface. The total current, or charge Q, as a function of time t could be expressed by the integrated Cottrell equation,[20,21]

$$Q = 2FnAC^{b}\left(\frac{Dt}{\pi}\right)^{1/2} + Q_{c} + Q_{ads} \quad (1)$$

where F is the Faraday constant (96 487 coulombs per equivalent), n the number of electrons involved in electrode reaction,

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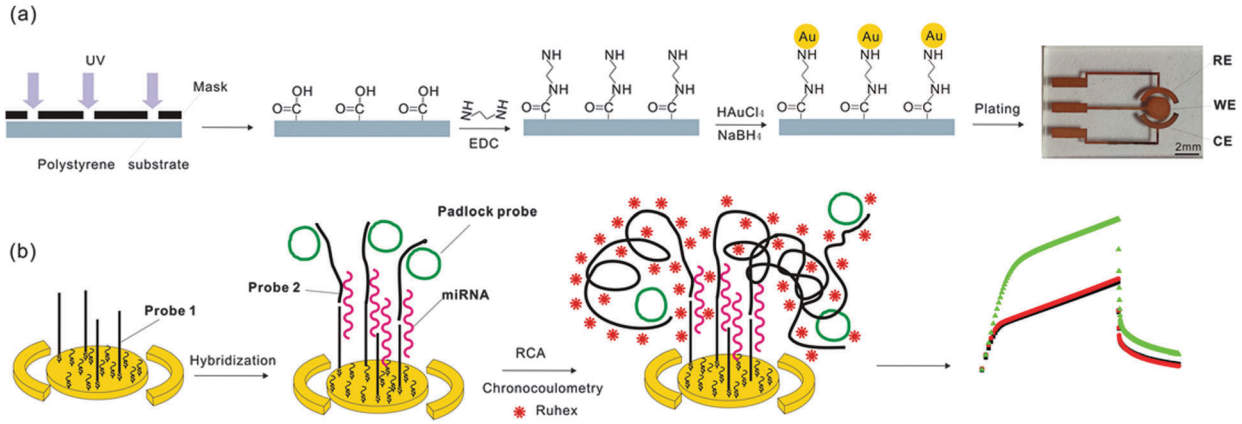

Fig. 1 Schematic illustration of the method. Integrated gold electrodes were fabricated on polystyrene substrate by chemical plating (a); Principle based on rolling circle amplification and Chronocoulometry (RCA–CC) for microRNA detection (b).

A the electrode area (cm^2), C^b the bulk concentration (mol cm^{-3}), D the diffusion coefficient (cm^2 s^{-1}), t the time (s), Q_c the capacitive charge (coulomb), and Q_{ads} the charge produced by the adsorbed reactant.

From Faraday's law, Q_{ads} in eqn (1) could be expressed as,

$$Q_{ads} = nFA\Gamma \quad (2)$$

where Γ is the quantity of the adsorbed reactant (mol cm^{-2}). The saturated surface excess of a redox marker (Ruhex) is converted to DNA probe surface density using the relationship,

$$\Gamma_{DNA} = \Gamma(z/m)(N_A) \quad (3)$$

where Γ_{DNA} is the probe surface density in molecules cm^{-2}, m is the number of bases in the probe DNA, z is the charge of the redox molecule (for Ruhex, z is 3), and N_A is Avogadro's number. By using ruthenium(III) hexaammine (RuHex), a cationic redox marker which could be electrostatically trapped onto the phosphate groups of the DNA probe, chronocoulometry is able to give a direct signal proportional to the number of phosphate groups of nucleic acids at the electrode surface. Based on eqn (3), a similar strategy could be used for quantification of single stranded and duplex DNA as well as RCA products (Fig. S1, ESI†). We investigated the behaviour of DNA as well as solid-phase RCA products on the gold electrode by chronocoulometry and cyclic voltammetry (Fig. S2, ESI†). Moreover this strategy provides a potential approach for absolute determination of probe density on the electrode surface under a reasonable assumption that when Ruhex is the only cation present in appreciable concentration (such as 50 μM), complete saturation of electrostatic adsorption between phosphate groups and Ruhex is achieved. Based on this strategy, we quantitatively investigated the immobilization, hybridization and RCA amplification on a gold electrode surface (Fig. 2). When a DNA probe 1 modified working electrode is hybridized with 10 nM of target microRNA and DNA probe 2, we could get a little increase of the chronocoulometry signal. While after four-hour RCA amplification, an obvious increase was observed due to the adsorption of Ruhex to RCA products on the electrode surface.

Based on eqn (2) and (3), we could calculate the surface density of DNA probe 1 immobilized on the working electrode ranging from

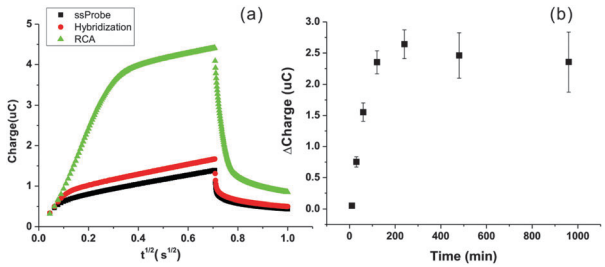

Fig. 2 Chronocoulometry plot of immobilization of a single stranded DNA probe on gold electrodes, hybridization with 10 nM of target molecules and four-hour RCA amplification (a); investigation of reaction time of RCA amplification including 10, 30, 60, 120, 240, 480 and 960 minutes respectively (b). ΔCharge is the difference in charge between RCA amplification and hybridization.

1×10^{12} to 6×10^{12} molecules per square centimetre (Fig. S3, ESI†). About 10% of probe 1 ($\sim 5.17 \times 10^{10}$ molecules) was observed to be hybridized with target microRNAs in the presence of 10 nM of mir-143 and probe 2. We investigated the amplification time to range from 10 minutes to 16 hours (Fig. 2b). RCA reaction was observed to occur at a rapid rate before 2 hours, while after four-hour amplification the products detected by chronocoulometry did not increase any more. In contrast, the signal got lower slightly because of non-specific amplification of the control. Moreover, we further calculated how many nucleotides have been produced after RCA amplification and estimated the reaction rate approximately. In our experiment, we found that the electrochemical signal was proportional to amplification time within one hour and the average reaction rate obtained was about nine nucleotides per minute which is much lower than 53 nucleotides per second reported by Lizardi *et al.*,[23] indicating that the reaction efficiency of RCA amplification on a solid surface is much lower than that in solution.

Mir-143 is proven to be a typical microRNA which targets the KRAS oncogene and acts as a tumour suppressor for a variety of cancers.[24] We chose mir-143 as target microRNA to demonstrate the principle of our method. Sensitivity of this RCA–CC assay was examined by using stepwise diluted target mir-143 ranging from 10 fM to 10 nM on the surface of gold electrodes. Although microRNAs could be detected by hybridization only at high

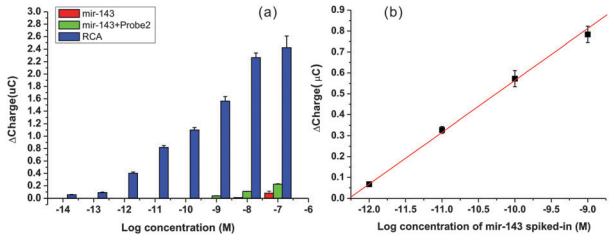

Fig. 3 Sensitivity of RCA–CC assay for detection of mir-143. Stepwise diluted target microRNAs were placed on the DNA modified gold electrodes. The concentration of mir-143 ranged from 10 fM to 100 nM. The concentration of DNA probe 2 added for initiation of RCA amplification was 10 nM and RCA reaction was performed at 30 °C for two hours (a); (b) detection of mir-143 spiked in human blood samples. The concentration of total RNA placed on the gold electrodes was ~500 pg μl^{-1}. ΔCharge is the difference in charge before and after RCA amplification.

concentration (100 nM), the sensitivity of hybridization detection could be improved to ~1 nM by adding DNA probe 2 (43 nt) in addition to microRNA. After RCA amplification, the directly measured detection limit was 100 fM, and the signal gain is linear over the range from 100 fM to 1 nM (Fig. 3a and Fig. S4, ESI†). The sensitivity of RCA–CC assay is similar with another chronocoulometry assay which employed gold nanoparticle for signal amplification.[25] However, it seems to be much lower than the fluorescent detection method.[5] In the future study, we could further improve the sensitivity by employing multiple amplification[26] or branched RCA.[6] Then, we investigated the specificity of this assay by exposing the immobilized electrode to three microRNAs (mir-1, mir-122 and mir-145) as well as mir-143 at the same concentration. The method exhibited good performance in terms of selectivity (Fig. S6, ESI†).

Subsequently, we investigated the practicality of this method to determine mir-143 in real world samples. It was reported that mir-143 is a biomarker of human prostate cancer and could be detected in blood as other circulating microRNAs.[27] A certain amount of synthesized mir-143 was spiked into healthy human blood samples, then total RNAs including spiked mir-143 were isolated and detected using RCA–CC assay. Without consideration of purification efficiency, as low as 1 pM of mir-143 in blood samples was detectable (Fig. 3b) and a dynamic range from 1 pM to 1 nM was obtained.

In conclusion, we developed a novel microchip containing an integrated gold electrode fabricated on a polystyrene substrate, which could be a promising biosensor. Unlike the traditional gold electrode fabricated on a glass substrate, which requires ultra-clean room and expensive instruments, this polymer electrode is of low cost and could be easily fabricated in an ordinary chemistry laboratory. Moreover, we developed a method for sensitive nucleic acid detection by combining solid-phase rolling circle amplification with chronocoulometry. Compared with other electrochemical methods, an attractive aspect of chronocoulometry is that it could perform absolute quantification of biomolecules on the surface of electrodes, therefore having great potential for molecular diagnostics and other applications, for example, the study of enzymatic activity at the molecular level.

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Notes and references

1 D. P. Bartel, *Cell*, 2009, **136**, 215–233.
2 J. Krol, I. Loedige and W. Filipowicz, *Nat. Rev. Genet.*, 2010, **11**, 597–610.
3 K. A. Cissell, S. Shrestha and S. K. Deo, *Anal. Chem.*, 2007, **79**, 4755–4761.
4 C. Hong, X. Chen, J. Li, J. Chen, G. Chen and H. Yang, *Chem. Commun.*, 2014, **50**, 3292–3295.
5 Y. Wen, Y. Xu, X. Mao, Y. Wei, H. Song, N. Chen, Q. Huang, C. Fan and D. Li, *Anal. Chem.*, 2012, **84**, 7664–7669.
6 B. Yao, J. Li, H. Huang, C. Sun, Z. Wang, Y. Fan, Q. Chang, S. Li and J. Xi, *RNA*, 2009, **15**, 1787–1794.
7 É. Várallyay, J. Burgyán and Z. Havelda, *Nat. Protoc.*, 2008, **3**, 190–196.
8 C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak and K. J. Guegler, *Nucleic Acids Res.*, 2005, **33**, e179.
9 E. Hamidi-Asl, I. Palchetti, E. Hasheminejad and M. Mascini, *Talanta*, 2013, **115**, 74–83.
10 H. Yin, Y. Zhou, H. Zhang, X. Meng and S. Ai, *Biosens. Bioelectron.*, 2012, **33**, 247–253.
11 H. Yang, A. Hui, G. Pampalakis, L. Soleymani, F. Liu, E. H. Sargent and S. O. Kelley, *Angew. Chem., Int. Ed.*, 2009, **48**, 8461–8464.
12 Y. Wen, H. Pei, Y. Shen, J. Xi, M. Lin, N. Lu, X. Shen, J. Li and C. Fan, *Sci. Rep.*, 2012, **2**, 867.
13 T. Goda, K. Masuno, J. Nishida, N. Kosaka, T. Ochiya, A. Matsumoto and Y. Miyahara, *Chem. Commun.*, 2012, **48**, 11942–11944.
14 Z. Ge, M. Lin, P. Wang, H. Pei, J. Yan, J. Shi, Q. Huang, D. He, C. Fan and X. Zuo, *Anal. Chem.*, 2014, **86**, 2124–2130.
15 Z. Gao, H. Deng, W. Shen and Y. Ren, *Anal. Chem.*, 2013, **85**, 1624–1630.
16 E. A. Lusi, M. Passamano, P. Guarascio, A. Scarpa and L. Schiavo, *Anal. Chem.*, 2009, **81**, 2819–2822.
17 T. Kilic, S. N. Topkaya, D. O. Ariksoysal, M. Ozsoz, P. Ballar, Y. Erac and O. Gozen, *Biosens. Bioelectron.*, 2012, **38**, 195–201.
18 Y. Peng, G. Yi and Z. Gao, *Chem. Commun.*, 2010, **46**, 9131–9133.
19 J. Wang, X. Yi, H. Tang, H. Han, M. Wu and F. Zhou, *Anal. Chem.*, 2012, **84**, 6400–6406.
20 F. C. Anson and R. A. Osteryoung, *J. Chem. Educ.*, 1983, **60**, 293–296.
21 A. B. Steel, T. M. Herne and M. J. Tarlov, *Anal. Chem.*, 1998, **70**, 4670–4677.
22 X. Hu, Q. He, H. Lu and H. Chen, *J. Electroanal. Chem.*, 2010, **638**, 21–27; Y. Kong, H. Chen, Y. Wang and S. A. Soper, *Electrophoresis*, 2006, **27**, 2940–2950.
23 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Genet.*, 1998, **19**, 225–232.
24 W. Gao, Y. Yu, H. Cao, H. Shen, X. Li, S. Pan and Y. Shu, *Biomed. Pharmacother.*, 2010, **64**, 399–408; X. Chen, X. Guo, H. Zhang, Y. Xiang, J. Chen, Y. Yin, X. Cai, K. Wang, G. Wang, Y. Ba, L. Zhu, J. Wang, R. Yang, Y. Zhang, Z. Ren, K. Zen, J. Zhang and C. Y. Zhang, *Oncogene*, 2009, **28**, 1385–1392.
25 J. Zhang, S. Song, L. Zhang, L. Wang, H. Wu, D. Pan and C. Fan, *J. Am. Chem. Soc.*, 2006, **128**, 8575–8580.
26 H. Ji, F. Yan, J. Lei and H. Ju, *Anal. Chem.*, 2012, **84**, 7166–7171.
27 P. S. Mitchell, R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 10513–10518.

